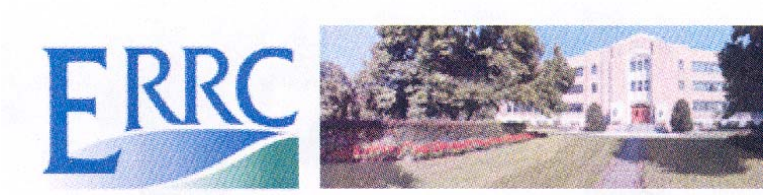

EASTERN REGIONAL RESEARCH CENTER
AGRICULTURAL RESEARCH SERVICE
UNITED STATES DEPARTMENT OF AGRICULTURE
600 E. MERMAID LANE
WYNDMOOR, PA 19038
(215) 233-6400

Title: Inactivation of Escherichia coli O157:H7 on Inoculated Alfalfa Seed with Ozonated Water Under Pressure

Author(s): R.R. Sharma, A. DeMirci, L.R. Beuchat and W.F. Fett

Citation: Journal of Food Safety (2002) 22: 107-119

Number: 7104

INACTIVATION OF *ESCHERICHIA COLI* O157:H7 ON INOCULATED ALFALFA SEEDS WITH OZONATED WATER UNDER PRESSURE[1]

RATNA R. SHARMA[2] and ALI DEMIRCI[2,3,4]

[2]*Department of Agricultural and Biological Engineering*
[3]*Life Sciences Consortium*
The Pennsylvania State University
University Park, Pennsylvania 16802

LARRY R. BEUCHAT

Center for Food Safety and Department of Food Science and Technology
University of Georgia
1109 Experiment Street
Griffin, Georgia 30223

AND

WILLIAM F. FETT

United States Department of Agriculture
Agricultural Research Service
Eastern Regional Research Center
Food Safety Intervention Technologies Research Unit
600 E. Mermaid Lane
Wyndmoor, Pennsylvania 19038

Received for Publication December 21, 2001
Accepted for Publication March 27, 2002

ABSTRACT

Alfalfa seeds inoculated with Escherichia coli *O157:H7 (~10^5 CFU/g) were subjected to low hydrostatic pressure. Seeds immersed in ozonated water at 4C were held at 8 and 12-psi ozone pressure for 2, 4, 8, 16, 32, and 64 min. Alternatively, seeds were continuously sparged with ozone for up to 64 min and then held at 12 psi for 5 min. Controls consisted of sparging and pressurization*

[1] Disclaimer: Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

[4] Author for correspondence: TEL: (814) 863-1098; FAX: (814) 863-1031; E-mail: demirci@psu.edu

with air. Thirty-two minute treatments of continuous ozone sparging followed by pressurization of seeds at 12 psi for 5 min were repeated with the addition of four surfactants (Tween 20, Tween 80, SPAN 20, and SPAN 80) in the treatment water. Enumeration of E. coli *O157:H7 on treated, untreated, and control seeds was done on tryptic soy agar supplemented with 50 μg/mL of nalidixic acid. The reduction in population of* E. coli *O157:H7 on seeds treated with the 8 and 12 psi hydrostatic pressure in ozonated water ranged from 0.74 - 1.56 log_{10} CFU/g and 0.72 - 1.62 log_{10} CFU/g, respectively. Control treatments carried out with air pressurization of seeds resulted in maximum population reductions of 1.55 log_{10} and 1.83 log_{10} CFU/g for 8 and 12 psi, respectively. For seeds treated with continuous ozone sparging (2 - 64 min) followed by pressurization at 12 psi for 5 min, the highest reduction was 2.03 log_{10} CFU/g. Reductions were, however, not significantly different ($P>0.05$) from control treatment (with air) which reduced the populations by 0.57 - 2.19 log_{10} CFU/g. The presence of surfactants during continuous sparging of water followed by pressurization at 12 psi was not beneficial. None of the treatments adversely affected the germination of the seeds.*

INTRODUCTION

Increase in consumption of raw sprouted seeds has led to a substantial rise in the occurrence of food poisoning outbreaks. The first documented outbreak of foodborne disease associated with sprouted seeds was recorded in 1973 (Portnoy *et al.* 1976). Over the years, many incidents have been reported worldwide. Between 1995 and 1998 there were nine major *E. coli* O157:H7 and *Salmonella* related outbreaks associated with commercial sprouts in the United States (NACMCF 1999).

Although the visible appearance of seeds is smooth, cracks or crevices on the surface or between the seed coat and cotyledon of damaged seeds are capable of harboring pathogens (Itoh *et al.* 1998; Charkowski 2001). Additionally, alfalfa seeds are sometimes subjected to an abrasion process known as scarification, which may facilitate diffusion of microbial cells into protected areas of the seed (Weissinger and Beuchat 2000). Apart from providing opportunities for pathogens to infiltrate seeds, these conditions offer protection against the active components of aqueous sanitizers. Therefore, contaminated seeds are the most likely source of pathogens (NACMCF 1999; Taormina *et al.* 1999) and sprouting provides a conducive environment for the growth of many microorganisms (Stewart *et al.* 2001).

A certification program, instituted by the International Sprout Growers Association, requires sprout producers to apply a seed disinfection treatment of 20,000 ppm $Ca(OCl)_2$ (NACMCF 1999). United States Food and Drug

Administration (Federal Register 1999) also recommends decontamination of seeds with 20,000 ppm of chlorine (using calcium hypochlorite). Regulatory agencies suggest that intervention strategies should achieve at least a 5-$\log_{10}$ reduction in pathogens such as *Salmonella* and *E. coli* O157:H7. Considerable research activity has therefore been directed towards seed decontamination (Beuchat 1997; Jaquette *et al.* 1996; Taormina and Beuchat 1999a, b; Weissinger *et al.* 2001). Though some chemical treatments have shown effectiveness in eliminating pathogens (Delaquis *et al.* 1999; Park *et al.* 2000) nevertheless, chemicals carry the risk of leaving behind carcinogenic residues that can be harmful to human health. Commercial requirements to extend the safe, high quality shelf-life of seed sprouts have thus focused attention on new methods and systems of decontamination.

Ozone is a potential alternative under investigation for its effectiveness in killing microorganisms on meat, poultry, eggs, fish, fruits, vegetables, and dry fruits (Kim *et al.* 1999). Ozone acts as a disinfectant in either the gaseous state or when dispersed in water, and is an effective biocide against viruses, bacteria, fungi, protozoa, and some other higher forms of life such as worms and mites. Preliminary experiments on the cocktail of *E. coli* O157:H7 strains used for this study have shown complete elimination of the pathogen within a minute of addition of ozonated water to culture broth. Ozone offers a number of advantages over conventional chemical disinfectant treatments such as chlorine. These include rapid dissociation to oxygen with a lack of residues or by-products after treatment. In addition, ozone offers a nonthermal disinfectant option suitable for seeds, sprouts, and leafy vegetables which are sensitive to deterioration of sensory quality upon exposure to steam and heat decontamination procedures. On June 26, 2001, FDA approved the use of ozone for treatment of raw commodities (Federal Register 2001).

In our previous study on ozone treatment to eliminate *E. coli* O157:H7 on alfalfa seeds, we observed that ozonated water at atmospheric pressure was a potential biocide resulting in population reductions greater than 2 $\log_{10}$ CFU/g (Sharma *et al.* 2002). However, we hypothesized that due to its inability to penetrate into damaged areas on the surface of the seeds, ozone at atmospheric pressure did not eliminate these pathogens completely. Mazzoni *et al.* (2001) applied up to 4000 psi pressure during treatment of alfalfa seeds with supercritical carbon dioxide. They concluded that applying pressure facilitates better penetration of the sanitizers into the inaccessible cracks and crevices of seeds thus enhancing microbial decontamination without compromising germination quality. The main advantage of application of hydrostatic pressure includes uniform transmission of pressure, regardless of the size and shape of sample (Mussa *et al.* 1999). Furthermore, Weissinger and Beuchat (2000) found that combination of aqueous sanitizers such as $Ca(OH)_2$ with a surfactant

enhanced inactivation of *Salmonella* on alfalfa seeds by promoting access of the lethal agent to the bacterial cells.

Therefore this study was undertaken to explore the potential benefits of applying secondary stress conditions, such as pressure and surfactants, simultaneously with ozone, to deliver the sterilant into inaccessible areas on the seeds surface thus resulting in greater reductions in bacterial populations. The antimicrobial effect of direct ozone sparging under low hydrostatic pressure, with and without the addition of surfactants in the treatment water was investigated. The effect of ozone on seed viability was also investigated to determine the feasibility of commercial application of pressurized ozone as a disinfectant in the sprout industry.

MATERIALS AND METHODS

Preparation of *E. coli* O157:H7 Inoculum

Five strains of enterohemorrhagic *E. coli* O157:H7 resistant to nalidixic acid were obtained from the Center for Food Safety, University of Georgia. The strains were: 932 (human isolate), 994 (salami isolate), E0018 (calf fecal isolate), H1730 (human isolate from outbreak associated with lettuce), and F4546 (human isolate from an outbreak associated with alfalfa sprouts). Cells were grown in tryptic soy broth (Difco, Detroit, Mich.) supplemented with 50 μg/mL nalidixic acid (Fisher Scientific, Fair Lawn, NJ) and 0.1% dextrose (TSBN) at 37C for 18 h. The use of nalidixic acid minimized growth of microorganisms other than *E. coli* O157:H7 in enumeration media. A mixture of the five *E. coli* O157:H7 strains was prepared by combining 100 mL of each 18-h culture and centrifuging (Sorvall STH750, Kendro Lab Product, Newtown, Conn.) at 4C and 3,300 $\times$ *g* for 15 min. The supernatant was decanted and the pellet was resuspended in 300 mL of sterile 0.1% peptone water before centrifuging again at 3,300 $\times$ *g* for 15 min at 4C. The pellet was then resuspended in one liter of sterile 0.1% peptone water.

Inoculation of Alfalfa Seeds

Alfalfa seeds (Lot No. AUS/A/DM 066) were obtained from International Specialty Supply (Cookeville, Tenn.). One kilogram of alfalfa seeds was soaked in one liter of the five-strain suspension of *E. coli* O157:H7 ($\sim 10^8$ CFU/mL) for 1 min with gentle agitation. After the inoculum was decanted, seeds were placed on a sterile perforated tray lined with four layers of cheesecloth and dried in a laminar flow hood at room temperature (21 $\pm$ 1C) for 24 h. Dried seeds containing $\sim 10^5$ CFU of *E. coli* O157:H7 per gram were sealed in plastic Ziploc® bags and stored at 4C until used within a week.

Production of Ozone and Measurement of Concentration

Ozone gas (0.34 m^3/h) was generated using a lab-scale ozone generator (Model No. H-50, Hess Machines International, Ephrata, Pa.) equipped with an oxygen concentrator. One liter of sterile, deionized water at an initial temperature of 4C in a polypropylene beaker placed in the pressure vessel was sparged with ozone through a 10 μm stainless steel sparger. The sparging time was varied with the type of pressure treatment. After treatment, excess ozone was passed through 2% potassium iodide solution to prevent ozone from being released into the environment. The sparging process was performed in a fume hood for safety purposes. The concentration of ozone in the water after the pressure treatment was determined by direct measurement of UV absorption at 258 nm (Sharma *et al.* 2002).

Pressure Vessel

A 15.5-quart (17.07 L) pressure sterilizer (Model No. 1915X, Wisconsin Aluminum Foundry Co. Inc., Manitowoc, Wisc.) was modified to make a pressure vessel suitable for treating seeds in ozonated water. Appropriate inlet and exit valves were provided to create and release pressure as desired.

Low Hydrostatic Pressure Treatment with Ozone

Twenty-five grams of contaminated alfalfa seeds immersed in one liter of sterile deionized water in a 1000 mL beaker were placed in the pressure vessel. The vessel was sealed and ozone gas was sparged through the water and seed mixture. Sparging with ozone was ceased when the desired pressure was reached. To investigate the effect of pressure on lethality of ozone to *E. coli* O157:H7, two pressure levels, 8 psi (55 kPa) and 12 psi (83 kPa), were studied. A pressure of 8 psi was attained by sparging ozone gas for 2.5 min, while sparging for 3.5 min was necessary to reach a pressure of 12 psi. Alfalfa seeds were held in ozonated water for 2, 4, 8, 16, 32, and 64 min at each hydrostatic pressure. Sterile deionized water sparged with air was included as a control.

Continuous Ozone Sparging Followed by 12 psi Hydrostatic Pressure Treatment

Ozone gas was continuously sparged through one liter of sterile deionized water containing 25 g of inoculated alfalfa seeds in the pressure vessel. After treating the seeds with ozone for 2, 4, 8, 16, 32, and 64 min, the exit valve of the vessel was closed and pressure was allowed to build to 12 psi within 3.5 min. Ozone flow was stopped and the pressure vessel was allowed to stand for 5 min before releasing the pressure and analyzing seeds. In the control, ozone was replaced with air.

Effect of Surfactant

To determine the effect of surfactant on inactivation of *E. coli* O157:H7 during continuous ozone sparging, followed by a 12-psi hydrostatic pressure treatment, Tween 20 (J.T. Baker, Phillipsburg, NJ) Tween 80 (Mallinckrodt Baker Inc., Paris, Ken.), SPAN 20, and SPAN 80 (Aldrich, Milwaukee, Wisc.) were evaluated. Tween 20 and Tween 80 were used in the treatment water at a concentration of 5 ppm, while SPAN 20 and SPAN 80 were used at 100 ppm. Seeds were sparged with ozone for 32 min, followed by pressurizing the vessel to 12 psi within 3.5 min, and allowing to stand for 5 min before releasing the pressure. Higher concentrations of surfactants caused excessive foaming during ozone sparging of the water and seed mixture, and were not investigated. The control consisted of ozone sparging for 32 min followed by pressurization to 12 psi and holding for 5 min. Ozone was not replaced with air since the variable for this treatment was surfactant and not ozone.

Microbiological Analysis

To determine the initial population of *E. coli* O157:H7 on seeds before treatment with ozone, 10 g of inoculated seeds were placed in 40 mL of sterile 0.1% peptone water in a Stomacher® 400 bag for 2, 4, 8, 16, 32, and 64 min. Seeds were treated with a Stomacher® for 30 s. The wash solution was serially diluted in sterile 0.1% peptone and surface plated (0.1 mL) in duplicate on tryptic soy agar (Difco) supplemented with 50 μg/mL nalidixic acid (TSAN). After incubating plates at 37C for 24 h, presumptive *E. coli* O157:H7 colonies were enumerated.

Populations of *E. coli* O157:H7 on ozone-treated seeds (with and without surfactant) were determined by placing the treated seeds (25 g) in 100 mL of sterile 0.1% peptone water followed by pummelling with a Stomacher® for 30 s, serially diluting in 0.1% peptone and surface plating on TSAN. Colonies formed on plates inoculated with peptone wash samples from untreated and treated seeds were randomly picked and subjected to a *E. coli* O157:H7 latex agglutination test (Remel Microbiological Products, Lenexa, Kan.) to confirm identity.

Effect of Ozone and Pressure Treatment on Viability of Alfalfa Seeds

Alfalfa seeds treated with ozone, surfactant and water were tested for viability, i.e., percent capable of germination. Approximately 100 seeds (treated and control) were placed between two moistened filter paper discs in a Petri dish. Water was applied by spraying from time to time over a 48-h period at 30C to provide sufficient moisture for viable seeds to germinate. Seeds were visually examined after 48 h, and the percentage that germinated was calculated.

Statistical Analysis

All treatments were replicated three times. The results were analyzed using MINITAB (Minitab Inc., State College, Pa.) for analysis of variance and determining significant and non-significant differences in $\log_{10}$ CFU/g of *E. coli* O157:H7 on alfalfa seeds subjected to each treatment. A 95% confidence level was used.

RESULTS AND DISCUSSION

Effect of Low Hydrostatic Pressure with Ozone on the Inactivation of *E. coli* O157:H7 and Viability of Alfalfa Seeds

The initial population of *E. coli* O157:H7 was ~ 5 $\log_{10}$ CFU/g of seeds. As the holding time of control seeds (unozonated water at atmospheric pressure) increased, the number of retrievable CFU increased. The concentration of ozone after treatment of inoculated seeds could not be measured since the water became turbid with the release of organic matter from the seeds and absorbance measured with the spectrophotometer gave an incorrect result. However, to estimate the amount of ozone in the water after treatment, sterile deionized water (without seeds) was continuously sparged with ozone with subsequent pressurization and the concentration of ozone was measured. The ozone concentration in water for 8 and 12 psi pressure treatments ranged from 8 - 17 ppm and 32 - 38 ppm, respectively, for 2 to 64 min sparging times. For continuous sparging followed by 12 psi pressure treatments, the concentration range was 20 - 26 ppm.

Holding inoculated alfalfa seeds in ozonated water at 8 psi reduced *E. coli* O157:H7 population by 0.74 $\log_{10}$ CFU/g within 2 min and by 1.56 $\log_{10}$ CFU/g within 64 min (Table 1). The control treatment, in which air was used to create a pressure of 8 psi, resulted in reductions in population ranging from 0.68 $\log_{10}$ CFU/g to 1.55 $\log_{10}$ CFU/g. Reductions on seeds treated with aerated water at 8 psi were not significantly different ($P > 0.05$). A similar trend was observed for seeds treated with ozonated water. Overall, at 8 psi, reductions in population of *E. coli* O157:H7 were not significantly different in aerated and ozonated water, regardless of treatment time.

Treatment of alfalfa seeds at 12 psi in aerated and ozonated water for up to 64 min reduced *E. coli* O157:H7 population by 1.83 and 1.62 $\log_{10}$ CFU/g, respectively (Table 1). The control treatment with air resulted in 1.83 $\log_{10}$ CFU/g reduction in *E. coli* O157:H7 population for a holding time of 32 min, which was significantly higher ($P \leq 0.05$) than treatment for 2, 4, or 8 min. The reduction in ozonated water was significantly lower for 2-8 min treatments.

At a given treatment time, reductions in aerated and ozonated water were not significantly different.

TABLE 1.
REDUCTION IN POPULATIONS OF *E. COLI* O157:H7 ON ALFALFA SEEDS TREATED IN AERATED AND OZONATED WATER UNDER PRESSURE FOR UP TO 64 MIN

Holding Time (min)	Population Reduction (log_{10} CFU/g)[1]			
	8 psi		12 psi	
	Air	Ozone	Air	Ozone
2	BC 0.76 A	B 0.74 A	BC 0.69 A	B 0.79 A
4	C 0.68 A	B 0.81 A	C 0.51 A	B 0.72 A
8	ABC 1.03 A	AB 0.98 A	BC 0.82 A	B 0.88 A
16	A 1.49 A	A 1.55 A	AB 1.36 A	AB 1.23 A
32	A 1.55 A	B 0.88 B	A 1.83 A	AB 1.41 A
64	AB 1.39 A	A 1.56 A	AB 1.37 A	A 1.62 A

[1] Reductions (log_{10} CFU/g) compared to number of *E. coli* O157:H7 recovered from untreated seeds soaked in peptone water for comparable times. Within the same pressure, values in the same row, not followed by the same letter are significantly different ($P \leq 0.05$). Within the same column, values not preceded by the same letter are significantly different ($P \leq 0.05$).

Analysis of variance using a general linear model involving two way interaction terms showed that there was no significant difference ($P > 0.05$) in the reduction of *E. coli* O157:H7 population by aerated and ozone treatments, both at 8 and 12 psi, with the exception of the 8-psi treatment at 32 min. This may be attributed to the very short ozone sparging times required to attain the desired pressure. Thus limited amounts of ozone would be dissolved in the treatment water and organic matter from the seeds would quickly consume all of the dissolved ozone.

Seeds not subjected to pressure or ozone treatment had 86% germination. The germination percentage for seeds was not significantly affected by treatment

($P > 0.05$) with air or ozone at 8 and 12 psi. Holding time also did not have any significant effect on the germination percentage. The percent germination of alfalfa seeds treated with air at 8 and 12 psi was in the range 78.8 - 85.1% and 82.4 - 92.5%, respectively. For the seeds treated with ozone, the germination percentages were 78.9 - 91.0% and 81.1 - 89.1% for 8 and 12 psi, respectively. There were no significant differences ($P > 0.05$) in germination rates for any of the treatments.

Effect of Continuous Ozone Sparging Followed by 12 psi Hydrostatic Pressure Treatment on Inactivation of *E. coli* O157:H7 and Viability of Alfalfa Seeds

Since reductions in populations of *E. coli* O157:H7 treated at 8 and 12 psi were not significantly different, continuous ozone sparging followed by pressurization was done only at 12 psi as this pressure was as easy to obtain as 8 psi.

Reductions in population of *E. coli* O157:H7 on seeds treated by continuous ozone sparging for up to 64 min and then pressurized at 12 psi for 5 min ranged from 0.67 to 2.03 $\log_{10}$ CFU/g (Table 2). There were no significant differences ($P > 0.05$) in reductions on seeds treated with ozonated water for 2, 4, 16, and 64 min, before pressurization. Continuous sparging with air and then pressure build up to 12 served as the control and resulted in a maximum population reduction of 2.19 $\log_{10}$ CFU/g at 32 min. The reductions for 2–8 min control treatments were not significantly different ($P > 0.05$) from each other. However they were significantly lower ($P \leq 0.05$) than 16 - 64 min treatment. At a given sparging time, there were no significant differences ($P > 0.05$) between the treatment in aerated and ozonated water.

There was no significant difference ($P > 0.05$) in percent germination of control or treated seeds, regardless of sparging time. The germination percentages for control and treated seeds were 77.3 - 87.6% and 80.1 - 86.5%, respectively.

Effect of Surfactant During Continuous Ozone Sparging Followed by Pressurization Treatment

Compared to treatment of seeds with aerated water, regardless of pressure, ozone application coupled with pressure did not increase the inactivation of *E. coli* O157:H7 on alfalfa seeds. This was probably due in part to the inability of the ozone to penetrate into the cracks and damaged surfaces of the seed coat under the pressure used in this study. In order to break the surface tension barrier and enable ozone to access subsurface areas of the seeds, surfactants were added to the water used for continuous ozone sparging for 32 min followed by pressurization at 12 psi for 5 min. Four surfactants (Tween 20, Tween 80,

SPAN 20, and SPAN 80) were evaluated (Table 3). The reduction in population was in the range of 1.37 - 1.51 $\log_{10}$ CFU/g. This range was significantly lower ($P \leq 0.05$) than the 2.03 $\log_{10}$ CFU/g reduction in *E. coli* O157:H7 population with control water not supplemented with a surfactant.

TABLE 2.
REDUCTION IN POPULATIONS OF *E. COLI* O157:H7 ON ALFALFA SEEDS TREATED BY CONTINUOUS SPARGING OF OZONE FOR UP TO 64 MIN FOLLOWED BY PRESSURIZATION AT 12 PSI

Sparging Time (min)[1]	Population Reduction ($\log_{10}$ CFU/g)[2]	
	Air	Ozone
2	C 0.63 A	BC 0.70 A
4	C 0.57 A	BC 0.73 A
8	C 0.66 A	C 0.67 A
16	AB 1.35 A	B 1.35 A
32	A 2.19 A	A 2.03 A
64	AB 1.75 B	B 1.92 A

[1] Sparging time at atmospheric pressure before pressurizing at 12 psi within 3.5 min, then holding at 12 psi for 5 min.

[2] Within the same row, values not followed by the same letter are significantly different ($P \leq 0.05$). Within the same column, values not preceded by the same letter are significantly different ($P \leq 0.05$).

There were no significant differences in germination percentage of seeds treated with and without surfactants. While the percent germination for control seeds was 84.8%, it ranged between 79.3 and 85.3% for treatments in water containing surfactants.

TABLE 3.
REDUCTION IN POPULATIONS OF *E. COLI* O157:H7 ON ALFALFA SEEDS AFTER TREATMENT BY CONTINUOUS SPARGING OF SURFACTANT SUPPLEMENTED WATER

Surfactant	Conc. (ppm)[1]	Population Reduction (log_{10} CFU/g)[2]
Control	0	A 2.03
Tween 20	5	B 1.41
Tween 80	5	B 1.37
SPAN 20	100	B 1.47
SPAN 80	100	B 1.51

[1] Concentration of surfactant in water continuously sparged with ozone for 32 min followed by pressurization at 12 psi within 3.5 min and holding at 12 psi for 5 min.
[2] Values not preceded by the same letter are significantly different ($P \leq 0.05$).

The significantly lower reduction in number of *E. coli* O157:H7 in ozonated water containing surfactants compared to the reduction in water not containing a surfactant may have been due to degradation of ozone with surfactants. Though the exact reaction mechanism is not known, such a situation would result in very little residual ozone to kill *E. coli* O157:H7 on the seeds.

Ozone has been used with varied success to inactivate microorganisms on a wide range of foods. Though ozone has been shown to be a strong antimicrobial agent for some treatments, this study did not demonstrate its lethality in eliminating *E. coli* O157:H7 on alfalfa seeds. Treatment with aerated water under pressure causes reductions in population similar to those achieved by treatment with ozonated, pressurized water. The application of pressures appears to be more promising than ozone in killing *E. coli* O157:H7 on alfalfa seeds. Ozone may have a synergistic effect when coupled with higher pressure but this is yet to be demonstrated.

ACKNOWLEDGMENTS

Funding for this project was provided by the National Alliance for Food Safety, the United States Department of Agriculture, Agricultural Research

Service (USDA Specific Cooperative Agreement No. 58-1935-0-022) and the Pennsylvania Agricultural Experiment Station. We also thank Hess Machine International (Ephrata, Pa.) for loaning the ozone generator used in this study.

REFERENCES

BEUCHAT, L.R. 1997. Comparison of chemical treatments to kill *Salmonella* on alfalfa seeds destined for sprout production. Intern. J. Food Microbiol. *34*, 329-333.

CHARKOWSKI, A.O., SARREAL, C.Z. and MANDRELL, R.E. 2001. Wrinkled alfalfa seeds harbor more aerobic bacteria and are more difficult to sanitize than smooth seeds. J. Food Prot. *64*, 1292-1298.

DELAQUIS, P.J., SHOLBERG, P.L. and STANICH, K. 1999. Disinfection of mung bean seed with gaseous acetic acid. J. Food Prot. *62*, 953-957.

Federal Register. 1999. Guidance for industry: reducing microbial food safety hazards for sprouted seeds and guidance for industry: sampling and microbial testing of spent irrigation water during sprout production. Fed. Regist. *64*, 57893-57902.

Federal Register. 2001. Secondary direct food additives permitted in food for human consumption, final rule. Fed. Regist. *66*, 338829-338830.

ITOH, Y. *et al.* 1998. Enteroheamorrhagic *Escherichia coli* O157:H7 present in radish sprouts. Appl. Environ. Microbiol. *64*, 1532-1535.

JAQUETTE, C.B., BEUCHAT, L.R. and MAHON, B.E. 1996. Efficacy of chlorine and heat treatment in killing *Salmonella stanley* inoculated onto alfalfa seeds and growth and survival of the pathogen during sprouting and storage. Appl. Environ Microbiol. *62*, 2212-2215.

KIM, J.G., YOUSEF, A.E. and DAVE, S. 1999. Application of ozone for enhancing the microbiological safety and quality of foods: A review. J. Food Prot. *62*, 1071-1078.

MAZZONI, A.M., SHARMA, R.R., DEMIRCI, A. and ZIEGLER, G.R. 2001. Treatment of alfalfa seeds with super critical carbon dioxide. J. Food Safety *21*, 215-223.

MUSSA, D.M., RAMASWAMY, H.S. and SMITH, J.P. 1999. High pressure destruction kinetics of *Listeria monocytogenes* on pork. J. Food Prot. *62*, 40-45.

National Advisory Committee on Microbiological Criteria for Foods (NACMCF). 1999. Microbiological safety evaluations and recommendations on sprouted seeds. Intern. J. Food Microbiol. *52*, 123-153.

PARK, C.M., TAORMINA, P.J. and BEUCHAT, L.R. 2000. Efficacy of allyl isothiocyanate in killing enterohemorrhagic *Escherichia coli* O157:H7 on alfalfa seeds. Intern. J. Food Microbiol. *56*, 13-20.

PORTNOY, B.L., GOEPFERT, J.M. and HARMON, S.M. 1976. An outbreak of *Bacillus cereus* food poisoning resulting from contaminated vegetable sprouts. Amer. J. Epidemiology *103*, 589–594.

SHARMA, R.R., DEMIRCI, A., BEUCHAT, L.R. and FETT, W.F. 2002. Inactivation of *Escherichia coli* O157:H7 on inoculated alfalfa seeds with ozonated water and heat treatment. J. Food Prot. *65*, 447–451.

STEWART, D.S., REINEKE, K.F., ULASZEK, J.M. and TORTORELLO, M.L. 2001. Growth of *Salmonella* during sprouting of alfalfa seeds associated with Salmonellosis outbreaks. J. Food Prot. *64*, 618–622.

TAORMINA, P.J. and BEUCHAT, L.R. 1999a. Behavior of enterohemorrhagic *Escherichia coli* O157:H7 on alfalfa sprouts during the sprouting process as influenced by treatments with various chemicals. J. Food Prot. *62*, 850–856.

TAORMINA, P.J. and BEUCHAT, L.R. 1999b. Comparison of chemical treatments to eliminate enterohemorrhagic *Escherichia coli* O157:H7 on alfalfa seeds. J. Food Prot. *62*, 318–324.

TAORMINA, P.J., BEUCHAT, L.R. and SLUTSKER, L. 1999. Infection associated with eating seed sprouts: An international concern. Emerging Infectious Dis. *5*, 626–634.

WEISSINGER, W.R. and BEUCHAT, L.R. 2000. Comparison of aqueous chemical treatments to eliminate *Salmonella* on alfalfa seeds. J. Food Prot. *63*, 1475–1482.

WEISSINGER, W.R., McWATTERS, K.H. and BEUCHAT, L.R. 2001. Evaluation of volatile chemical treatments for lethality to *Salmonella* on alfalfa seeds and sprouts. J. Food Prot. *64*, 442–450.